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Research paper

Immune polychemotherapy regimen choice in B-cell non-Hodgkin lymphoma of high and low malignancy based on the identification of the mutational c-myc and BCL 2 genes

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Abstract

Introduction: The relevance of research is conditioned by the study of the gene expression profile for the identification of molecular subgroups of non-Hodgkin B-cell lymphomas (NHBCLs) in haematology.

Aim: The aim of this research was to study the gene expression profile with the identification of molecular subgroups in patients with NHBCLs for personalised treatment.

Material and methods: This paper is aimed at analysing the frequency and role of expression of c-myc, B-cell lymphoma 2 (BCL 2) proteins and the Ki 67 proliferative index in patients with NHBCLs and conducting personalised therapy to improve the immediate effectiveness and immediate treatment results.

Results and discussion: The paper presents the results of the use of high-dose polychemotherapy (PCT) in 9 patients out of 80 with NHBCL during co-expression of the c-myc, BCL 2 mutational gene and with high values of the Ki 67 proliferative index. High-dose chemotherapy (HDCT) was performed according to the R+HyperCVAD scheme (6 courses) and hematopoietic stem cell (HSC) autotransplantation improved the immediate effectiveness of therapy, with a complete remission rate of 80% and an event-free survival of 28 months.

Conclusions: The study of molecular genetic characteristics in 80 patients with NHBCLs revealed co-expression of the c-myc and BCL 2 mutational gene in 9 out of 80 patients, and they differed in the aggressive course, 'poor' response to therapy, which predetermined the use of high-dose PCT with transplantation of autologous stem cells.

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1. INTRODUCTION

It was established that studying the gene expression profile for identifying the molecular subgroups of non-Hodgkin B-cell lymphomas (NHBCL) constitutes an urgent oncohaematologic problem, because a personalised approach to the treatment of patients with non-Hodgkin lymphoma (NHL) with the use of the high-dose chemotherapy regimen (HDCR), which includes a monoclonal antibody and subsequent autotransplantation of hematopoietic stem cells (autoGSC), allows to increase the immediate effectiveness of the therapy, immediate results and the event-free survival of patients. Based on clinical and laboratory parameters, NHLs are stratified according to the criteria of the international prognostic index (IPI) into low, intermediate, and high-risk groups, which correlates with overall survival (OS) and event-free survival (EFS).[1,2]

Among oncogenic events, in 5%–14% of cases of diffuse large B-cell lymphoma (DLBCL), a rearrangement of the c-myc gene is detected. In this case, the disease is described by an aggressive course and an unfavourable prognosis.[3] In 20%–30% of cases of DLBCL, chromosomal translocation t(14; 18)(q32; q21) is detected, combined with hyperactivation of the BCL 2 oncogene and increased accumulation of its product, protein BCL 2, which plays a significant role in suppression of apoptosis. Less common is the chromosomal translocation t(8; 14)(q24; q32) associated with the overexpression of the c-myc oncogene, which encodes a protein that also regulates proliferation, differentiation, and apoptosis. This anomaly is more inherent in Burkitt lymphoma, but is also detected in 5%–15% of cases of DLBCL. In NHBCL, in 17% of cases, BCL 2 gene rearrangement is detected, and in 20%–24% of cases – BCL 6.[4]

GCB-type NHBCL is described by dysregulation of increased expression of BCL 2 and, often, c-myc gene. The ABC type of DLBCL is accompanied by translocation involving the BCL 6 gene, and in some, by inactivation of p53. Patients with GCB-type lymphomas, on average, have both the best response to standard chemotherapy and the best prognosis.[5] To solve the problems of the scientific and technical program, it is necessary to identify and develop various biological markers, which can facilitate the study of the molecular biological characteristics of NHLs in more detail and conduct personalised treatment with consideration of the characteristics of the tumour in a particular individual. In this regard, in all planned scientific studies, the main purpose is to study the effectiveness of the molecular genetic profile of BCLs and thereby improve and implement innovative approaches in the diagnosis and treatment prognosis.[1,6]

2. AIM

The aim of this research was to study the gene expression profile with the identification of molecular subgroups in patients with NHBCLs for personalised treatment.

3. MATERIAL AND METHODS

A scientific study was conducted on 80 primary patients with II–IV NHBCL at A and B stages of the disease. In all cases, the diagnosis was verified by histological and immunohistochemical (IHC) research methods, after which all patients underwent fluorescence in situ hybridization (FISH) studies and, factoring in the data of molecular genetic studies, the patients were divided into a high and low risk group and underwent personalised immune polychemotherapy followed by autotransplantation of hematopoietic stem cells (HSCs).

Inclusion criteria were primary patients over 18 years old with a verified diagnosis of BCL, histological, and IHC studies (BCL 2), proliferative activity (Ki 67) and the stage of the tumour process. Patients with status established on the Eastern Cooperative Oncology Group (ECOG) scale with the number of points. Other inclusion criteria are as follows:

(1) absolute neutrophil count more than or equal to $1 \times 10^3/\mu L$,
(2) platelet count more than or equal to $50 \times 10^3/\mu L$,
(3) serum creatinine and urea less than or equal to $1.5\times$ the upper limit of normal,
(4) ALT and AST less than or equal to $2.5\times$ the upper limit of normal.

All patients underwent computed tomography (CT) of the chest organs, magnetic resonance imaging (MRI), and ultrasound investigation of the abdominal cavity, positron emission tomography combined with CT (PET-CT) to record remission.

All patients with BCLs underwent a molecular genetic study to identify the c-myc genetic mutation (8; 14), BCL 2, and the Ki 67 proliferative index for personalised therapy.

Division of patients into high and low risk groups and conducting chemotherapy courses based on the results of cytogenetic studies.

It is planned to conduct high-dose PCT in patients with high-grade NHBCL with co-expression of the c-myc and BCL 2 gene according to the R+Hyper-CVAD regimen (6 courses), including immunotherapy (rituximab 375 mg/m^2, 1 day, cyclophosphamide – CF 300 mg/m^2, 2 times a day, 2–4 days, mesna 600 mg/m^2, 2–4 days, doxorubicin 16.7 mg/m^2 i.v. 72-hour infusion, 5–7 days, vincristine 1.4 mg/m^2, on 5th and 12th days, dexamethasone 40 mg i.v., 2–5 and 12–15 days) and HSC autotransplantation.

Patients with low-grade NHBCL with an undetected c-myc gene, but with a positive BCL 2 gene and a high proliferative Ki 67 index, are scheduled for PCT according to the usual regimen and R+CHOEP, with the inclusion of the monoclonal antibody rituximab (rituximab 375 mg/m^2, 0 day, cyclophosphamide 675 mg/m^2, 1 day, doxorubicin 40 mg/m^2, 1 day, vincristine 1.4 mg/m^2 i.v., 1 day, prednisone 40 mg/m^2 i.v. 1–5 days), or R-CHOP (rituximab 375 mg/m^2, 0 day, cyclophosphamide 675 mg/m^2, 1 day, doxorubicin 50 mg/m^2, 1 day, vincristine 1.4 mg/m^2 i.v. 1 day, prednisone 40 mg/m^2 i.v. on 1–5 days, etoposide 100 mg/m^2 i.v. on 1–3 days).

For negative values of BCL 2 and with a low proliferative index Ki 67 without an identified mutational

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gene, PCT was performed according to the usual R+CHOP scheme.

Evaluation of therapy efficacy according to clinical, laboratory, and instrumental studies (complete blood count – CBC, lactate dehydrogenase – LDH, alkaline phosphatase – ALP, radiographic, ultrasound, CT, MRI and PET-CT data, etc.).

FISH is a cytogenetic method used to detect and determine the position of a specific DNA sequence on metaphase chromosomes or in interphase *nuclei in situ*. The FISH method is used in preimplantation, prenatal and postnatal genetic diagnosis. Using FISH allows you to identify various chromosomal abnormalities: deletions, translocations, amplifications, etc. The FISH method is based on the use of fluorescently labelled DNA probes, which constitute artificially synthesised DNA fragments (oligonucleotides), the sequence of which is complementary to the DNA sequence of the studied aberrant chromosomes.

The study is divided into two main stages: denaturation (the process of obtaining single-stranded DNA sequences) and hybridisation (the reaction of combining single-stranded DNA sequences of the probe and the DNA of the sample with the formation of double-stranded molecules). Hybridisation was performed with the use of the Vysis MYC Break-Apart FISH Probe Kit and the Vysis Paraffin Pretreatment Reagent Kit (Abbott, United States, Texas). The signals were evaluated with the use of an AXIO IMAGER 2 fluorescence microscopes (Carl Zeiss, Jena, Germany) with a 100× magnification with a triple filter (Green/Orange/DAPI). Signals were counted in at least 50 nuclei, in 4 different areas of the tumour. The presence of fusion signals (green and orange) did not have clinical significance. The detected c-myc rearrangement during the diagnosis of BCL (according to published data) leads to a worsening of prognosis and requires an aggressive approach to the first line of therapy.

Primary assessment points included: the size of the primary tumour and its localisation according to X-ray tomographic studies, CT of the abdominal cavity and chest, MRI, as well as ultrasound of peripheral and retroperitoneal lymph nodes, CBC, a biochemical blood test (LDH and ALP), IHC of the tumour substrate, myelogram, cytogenetic data, immunological studies and patient activity according to the ECOG scale.

Secondary assessment endpoints included: the degree of tumour regression (complete, partial regression) based on data on tumour size reduction, relief of symptoms of intoxication, improvement of X-ray, CT, MRI, PET-CT, ultrasound, CBC and biochemical studies (LDH and ALP), myelogram data after 4–6 courses of PCT and the degree of activity on the EGOC scale, as well as the event-free survival of patients.

4. RESULTS

To solve the problems of the scientific and technical programme, it is necessary to identify and develop various biological markers, which can facilitate the study of the molecular biological features of NHLs in more detail and conduct personalised treatment with consideration of the characteristics of the tumour of a particular individual. In this regard, in all planned scientific studies, the main purpose is to increase the efficacy of the molecular genetic profile of a tumour of the lymphoid system and thereby improve and implement innovative approaches in the diagnosis and treatment prognosis.[1]

4.1. The features of the treatment of patients with III–IV NHBCL at A and B stages based on the study of the c-myc and BCL 2 mutational genes

The authors analysed the results of treatment of 80 patients with II–IV BCLs at A and B stages, located in the centre of haematology and bone marrow transplantation. The distribution of patients according to the histological type was as follows: follicular DLBCL – 19 patients, primary mediastinal DLBCL – 8; mantle zone DLBCL – 12; mucosa-associated lymphoid tissue (MALT) lymphoma – 12; basal border DLBCL – 9; Burkitt's lymphoma – 6; brain DLBCL – 6; lymphoblastic – 8 patients. The distribution of patients with NHBCL according to the stages of the disease was as follows: II at A and B stages comprised 21 patients, III at A and B stages – 46 patients, and IV at A and B stages – 13 patients. The age of patients ranged from 27 to 58 years, the average age was 42 years; 34 men, 46 women. In all cases, BCL was confirmed, IHC study found the expression of CD 20 antigen in all patients.

According to molecular genetic studies, in 9 out of 80 patients, the c-myc and BCL 2 mutation gene (co-expression) were identified in tumours, in which Ki 67 had high values, varied from 70% to 90.0%, averaged 83.0+4.6%, i.e., patients tended to have an aggressive course and a 'poor' response to the therapy, which predetermined the use of high-dose PCT, including the monoclonal rituximab antibody and subsequent autotransplantation of HSCs. Of the 80 patients, in 71 cases, the mutational c-myc gene was not identified and those patients were assigned to the tumour group with a low degree of malignancy. The average Ki 67 values in such categories of patients ranged from 60.0% to 76.0%, in these tumours the BCL 2 gene was always positive. For low Ki 67 values – the range was of 35%–57%, and BCL 2 was not found in these tumours (Table 1).

According to Hu et al.[7] when R-CHOP regimens were used in these patients, the 3-year OS was 43% and 86%, and progression-free survival (PFS) was 39% and 75%, respectively. It was first described in the early 1980s with follicular lymphoma. A functionally active protein encod-

Table 1. Ki 67 and BCL 2 gene expression level in patients with NHBCLs.

Ki 67 thresholds in NHBCL, %	BCL 2 gene expression in NHBCL
70.0 – 90.0 (high)	positive
60.0 – 76.0 (average)	positive
35.0 – 57.0 (low)	positive

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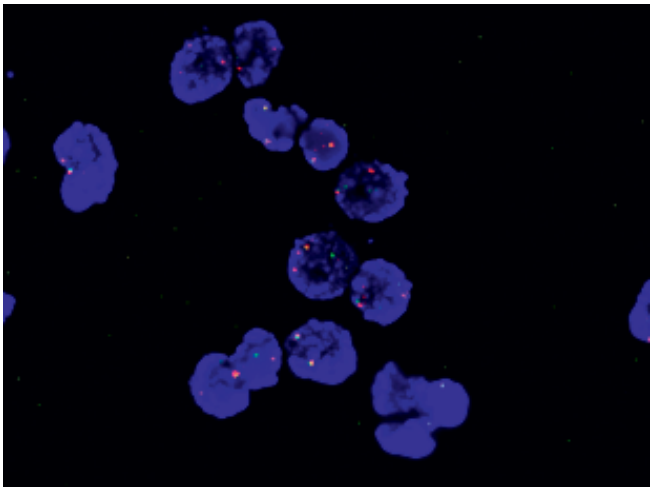

Figure 1. The result of the FISH study of patient M., 56 years old.

ed by this gene has two isoforms. The first isoform comprises 205 amino acid residues, and is a globular protein. The second isoform is described by the presence of an additional hydrophobic site of 34 amino acid residues, and designed to fix the protein in the mitochondrial membrane. Both isoforms are structurally similar to each other. The main difference between BCL 2 protein and BCL 6 and MYC lies in the structure of the substrate. If BCL 6 and MYC are DNA-binding regulatory factors, then BCL 2 binds to other proteins. BCL 2 functions as an anti-apoptotic factor: binds and inactivates the pro-apoptotic Bax protein. Furthermore, BCL 2 forms a complex with the apoptosis factor APAF1, preventing its ability to activate the caspase-dependent apoptosis pathway [123; 125; 198; 205]. The results of the FISH study are presented in Figure 1. Vysis DNA probes RUNX1/RUNX1T1DF: 2G 2O.Vysis DNA probes IGH/MYC/CEP 8 2G2O2A.

Nine patients out of 80 with III–IV NHBCL at A and B with co-expression of the c-myc gene and BCL 2 received a high-dose PCT according to the R+HyperCVAD regimen of 6 courses, then all patients underwent HSC autotransplantation in the amount of 4.0–4.8 million stem cells. Of 9 patients, 4 patients were with primary DLBCL brain lesion, 4 patients – with Burkitt lymphoma, and 1 patient with mediastinal NHBCL. The result of therapy demonstrated that by the end of the 6th course of PCT in 4 patients with Burkitt lymphoma, partial regression (PR) of IV NHBCL at B stage of more than 80.0% of the tumour process was recorded; in 1 patient with a mediastinal form of NHBCL, complete regression (CR) of the tumour process was recorded, including conglomerate of mediastinal lymph nodes, a decrease in compression syndrome and relief of intoxication and pain symptoms. In the remaining 5 patients, partial regression of the process was recorded (by 75.0%–80.0%), including relief of symptoms of intoxication and respiratory failure. In 4 patients with primary cerebral NHBCL, a regression of the main focus was established by 70% compared with its initial size, and peripheral lymph nodes by 80%. Bone marrow was intact.

After the treatment, all patients with the mutational c-myc gene and BCL 2 underwent a control examination of the bone marrow. It was found that in all cases, when examining the bone marrow in patients, bone marrow damage was not recorded. The activity of patients after treatment according to the ECOG-WHO system was 1 point. Laboratory data show that in all cases, relief of symptoms of intoxication, anaemia, and a decrease in ESR to an average of 18 mm/h were found. It was found that according to Table 1, prior to treatment, in patients with DLBCL in both groups, protein-forming and detoxifying liver functions are significantly affected. In patients before treatment, hypoproteinaemia is noted, which after treatment in the main and control groups increases equally by an average of 10 g/L. The indicators of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and creatinine were quite high, after treatment, they decreased by an average of 3 times compared with the control group, and such indicators of a biochemical blood test as LDH and ALP drop by 2–3 times compared to their initial indicators ($P = 0.05$) (Table 2).

After treatment, there is a positive tendency in biochemical blood tests of patients. Thus, total bilirubin was 13.1 μmol/L, total protein – 65 g/L, creatinine – 80 mmol/L, LDH – 131 mmol/L, ALP – 85 mmol/l, and ALT and AST are within acceptable limits. With a repeated myelogram after 3 courses of PCT, bone marrow damage was not registered. A side effect of PCT was expressed in the form of deep cytopenia, febrile neutropenia, and emetic syndrome, which stopped after symptomatic therapy. According to ECOG-WHO, quality of life scored 1 point.

Example. Patient A, 47 years old, clinical diagnosis: NHL, IV B stage with damage to the lymphatic collectors above and below the diaphragm. The patient underwent 6 courses of PCT according to the R+HyperCVAD regime, followed by autotransplantation of HSCs in an amount of 4.8 million stem cells, partial remission (more than 80%) of all groups of peripheral lymph nodes and over 70% of the formation of bronchopulmonary lymph nodes, as well as retroperitoneal lymph nodes were achieved. The patient is scheduled to continue chemotherapy according to the specified mode. According to ECOG-WHO Performance Status, quality of life after treatment scored 1 point. EFS in all patients was more than 28 months (Figures 2–3).

Table 2. Indicators of a biochemical blood test of patients after PCT (R+HyperCVAD).

Indicators	Main group	
	Before treatment	After 3 courses
Protein, g/L	56.5 + 3.4	65.0 + 4.6
ALT, U/L	7.8 + 0.8	1.76 + 0.08
AST, U/L	6.6 + 0.05	1.9 + 0.03
LDH, M/unit	356 + 4.3	116 + 12.9
Alkaline phosphatase, U/L	198.0 + 2.0	80.0 + 4.2
Creatinine, μmol/L	130.0 + 2.8	80.0 + 0.8

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4.2 The specifics of the treatment of patients with II–IV BCLs at A and B stages without detection of the c-myc mutational gene

This group includes 71 patients with II–IV NHBCLs at A and B stages without the identified c-myc mutation gene, with the expression of the BCL 2 gene and Ki 67 protein. Of these, 37 patients had NHL of high grade of malignancy NHBCL where Ki 67 ranged from 80% to 100% and BCL 2 was positive, and the remaining 34 patients showed an indolent form of NHBCL, Ki 67 ranged from 35% to 57%, BCL 2 was negative. A study of the myelogram showed that all patients with high-grade NHBCL had bone marrow damage, whereas the same was not established in patients with low-grade NHBCL. The results of treatment of 37 patients with NHBCL without expression of the c-myc mutation gene with high Ki 67 values (87.0%) and with BCL 2 gene expression during 6 courses of PCT according to the R-CHOEP 21 scheme showed that in this category of patients' complete remission of the tumour was achieved in 70.0+6.6%, patients partial – in 30.0+20.0% of patients, disease progression was not registered.

In 34 patients with a low degree of aggressiveness of II–IV BCLs at A and B stages, 6 courses of PCT according to the R+CHOP 21 scheme were performed to consolidate the process. In this case, complete remission of the tumours was achieved in 29 patients (80.0+10.0%), partial remission – in 8 patients (20.0+5.3%), there was no progression of the process during the treatment period. It can be assumed that the effectiveness of immune polychemotherapy of NHBCL with a low proliferative index is higher than that of patients with a high proliferative index. Thus, it can be assumed that preliminary results of treatment of 9 patients with DLBCL with the identified c-myc and BCL 2 genes treated according to R+HyperCVAD regimen, which includes immunotherapy and allows to conclude on the effectiveness of high-dose chemotherapy, the tumour's complete response to therapy was 80.0 %, EFS was more than 28 months.

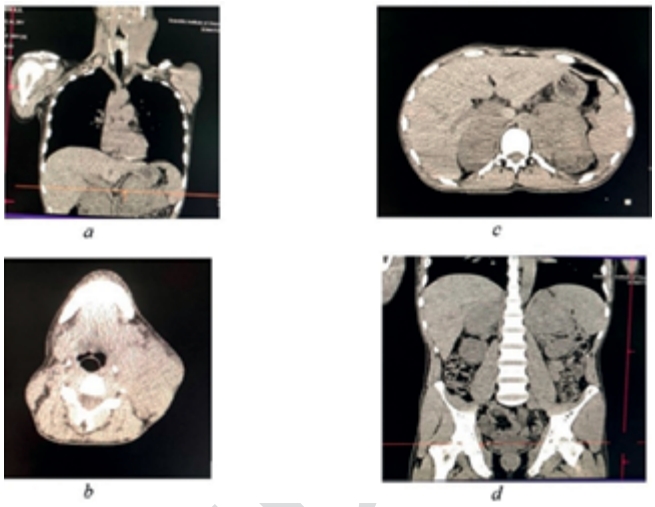

Figure 2. Multispiral CT before treatment of patient M, 56 years old: (a) Multispiral CT of the chest area before treatment; (b) Multispiral CT of the neck before treatment; (c) Multispiral CT of the abdominal cavity before treatment; (d) Multispiral CT of the abdominal cavity before treatment.

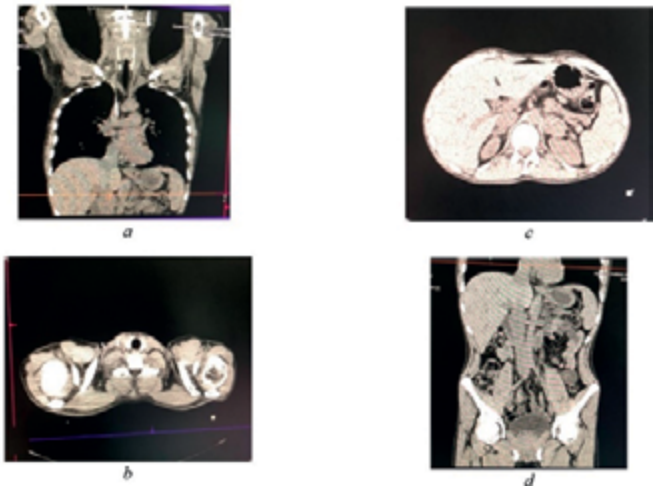

Figure 3. Multispiral CT after the treatment of patient M: (a) Multispiral CT of the neck after the treatment; (b) Multispiral CT of the chest area after the treatment; (c) Multispiral CT of the abdominal cavity before treatment; (d) Multispiral CT of the abdominal cavity before treatment.

5. DISCUSSION

According to modern concepts, based on the results of a study of the gene expression profile, there are several subtypes of DLBCL: from B-cells of the germinal centre and from activated B-cells. The c-myc, BCL 6, and BCL 2 genes are key regulators of the development of B-lymphocytes at the level of germinal (follicular) differentiation. It is possible that the determination of anomalies in the c-myc, BCL 6, and BCL 2 genes, as well as quantitative parameters of the expression of the proteins encoded by them, will facilitate the identification of risk groups among patients with DLBCL. In DLBCL, c-myc gene rearrangement occurs in approximately 5%–14% of cases.[8,9] As was previously shown, c-myc rearrangement leads to an aggressive course of the disease and poorer survival of patients with DLBCL. Thus, according to the literature, in most cases, DLBCL with c-myc rearrangement manifests itself as extranodal lesions, a late stage of the disease (stage III–IV according to Ann-Arbor classification). Tumour cells are described by a high proliferative index Ki-67 (>80%). Patients have lower OS, the worst response to R-CHOP therapy compared to cases without c-myc restructuring. The frequency of relapse of the disease involving the central nervous system increases.[8,10]

Similar to our study, the research by Hu et al.,[7] upon comparing the results of immune histochemical studies, FISH, and determining the gene expression profile with clinical data, it was proven that cases of DLBCL with co-expression of MYC/BCL 2 (threshold value of 40% for MYC, 70% for BCL 2) have the most unfavourable treatment outcomes. Green et al.[11] in a group of 193 patients with DLBCL showed that the co-expression of MYC/BCL 2 (threshold values of more than 40% and

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70%, respectively) correlated with an unfavourable prognosis of the disease upon the use of the R-CHOP regimen (3-year partial survival 43% vs. 86%; PFS 39% vs. 75%). In the MYC/BCL 2 co-expression group, 54% of patients had genetic abnormalities of the corresponding genes according to the results of the FISH study. The prognostic effect of MYC/BCL 2 co-expression was statistically significant when other factors were considered (e.g., IPI, belonging to the GCB/non-GCB subtype, the presence of c-myc/BCL 2 rearrangements).[12–15]

The author's study of the molecular genetic features of BCL revealed a biological variety and identified several genetic subtypes of this disease, which differ in course and prognosis. Subtypes of B-cell lymphoma develop from genetic disorders in the course of maturation of B-lymphocytes and are described by blocking apoptosis, impaired regulation of BCL 2, loss of function of BCL 6, deletion of p53 and increased cell proliferation, impaired regulation of c-myc. GCB-type NHBCL is described by dysregulation of increased expression of BCL 2 and, often, c-myc gene. The ABC type of DLBCL is accompanied by translocation with the involvement of the BCL 6 gene, and in some it is accompanied by inactivation of p53. Patients with GCB-type lymphomas, on average, have both the best response to standard chemotherapy and the best prognosis. We believe that in cases of aggressive BCL, NF-kB is activated in the classical way and c-myc regulation is impaired, which leads to refractory and to standard chemotherapy, and can be overcome by prescribing high-dose chemotherapy, including a monoclonal antibody (R+HyperCVAD).

The empirical approach to the choice of anticancer therapy is becoming obsolete. New knowledge about the functioning of the tumour cell, the identification of targets for the effects of drugs, the lack of effectiveness of cytostatics motivate to search for ways to individualise specific therapy based on molecular markers. As a result, the main emphasis in the paper is made on the analysis of chromosomal changes in tumour elements, without which modern diagnostics of blood diseases of a tumour nature, assessment of their prognosis, and choice of rational therapy are either impossible or fraught with big errors.

The study of the molecular genetic features of DLBCL c-myc and BCL 2 allowed many authors to identify biological heterogeneity and identify several genetic subtypes of this disease, which differ in course and prognosis. In this regard, in all planned scientific studies, the main purpose is to study the effectiveness of the molecular genetic profile of a tumour of the lymphoid system and thereby improve and implement innovative approaches in the diagnosis and treatment prognosis.[16–20] The results of a study of the molecular genetic features of BCL revealed the presence of the c-myc and BCL 2 gene in 9 patients and 80 co-expressions and the use of high-dose immune chemotherapy allowed to identify several genetic subtypes of this disease that differ in the course and response to therapy. The use of high-dose PCT followed by HSC transplantation in patients with co-expression of the c-myc and BCL 2 gene showed a high efficiency of the therapy and justified our prognosis of treatment effectiveness.

5. CONCLUSIONS

The authors revealed a correlation between the proliferative activity of the tumour (Ki 67) and the detection of the c-myc gene. Application of high-dose PCT according to the R+HyperCVAD scheme (6 courses) in patients with a mutational gene improved the immediate therapy efficacy, the frequency of complete remission is 80%, the EFS is over 28 months. Unprecedented survival in the group of patients with co-expression of c-myc and BCL 2 genes is 28 months.

Conflict of interests
None declared.

Funding
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